Abstract

Organisms that are invisible to the naked eye are the most abundant component of any freshwater community. These tiny organisms span domains and phyla and include viruses, prokaryotes (archae and bacteria), protists (single-celled eukaryotes such as single-celled fungi, algae, and protozoans) and multicellular fungi and microscopic metazoans (such as nematodes). This chapter gives an overview of microscopically small organisms, including their contribution to biodiversity, and techniques used by biologists studying these organisms in fresh waters, such as sampling regimes and methods used in the laboratory. To conclude, three case studies are given which had a common aim: to estimate the abundance and activity of microscopically small organisms in natural, freshwater communities.

Keywords

virus, bacteria, fungi, protozoan, microscopic metazoan, phage, protist, microbe, meiofauna, zooplankton

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Microorganisms 2:

Viruses, prokaryotes, fungi, protozoans, and microscopic metazoans

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8.1 Introduction

Organisms that are invisible to the naked eye are the most abundant component of any freshwater community. These tiny organisms span domains and phyla and include viruses, prokaryotes (archae and bacteria), and protists (single-celled eukaryotes such as single-celled fungi, algae—see Chapter 7, and protozoans), as well as multicellular fungi and microscopic metazoans (such as nematodes and rotifers). Microscopically small organisms do not only exceed macroscopic eukaryotes in terms of their numbers by far, they are also extremely bio-diverse (Green and Bohannan 2006) and contribute substantially to energy flows in freshwater ecosystems. For example, it is estimated that, globally, heterotrophic biota in inland waters respire 1.2 Pg of terrestrial derived carbon each year and release it to the atmosphere (Battin et al. 2009) and as a very rough approximation more than two-thirds of this carbon is respired by heterotrophic microbes, if we assume that production (the turnover of matter over time) is scale invariant with body mass and consider a typical size distribution in fresh waters.

Microscopically small organisms drive the bulk of ecosystem processes on this planet and techniques for estimation of their biodiversity, their sampling, and calculation of community processes, such as production, are vital if we want to assess ecosystem health and functioning of fresh waters. Here I give an overview of microscopically small organisms and techniques used by biologists studying these organisms in fresh waters (other than algae and biofilm, see Chapter 7). Because the organisms discussed here span domains of life and a plethora of phyla and functional groups (i.e., extensive differences exist when it comes to techniques for assessing them) I have highlighted literature that has many relevant references cited within and that will help with further reading (see also Tables 8.1 and 8.2). It is helpful to read this chapter in conjunction with Chapter 7, where a number of generic techniques have been described for surveying and sampling microorganisms and to consult the textbooks 'Freshwater Microbiology' (Sigee 2004), 'Wetland Techniques' (Anderson and Davis 2013), and 'Methods in Stream Ecology' (Hauer and Lamberti 2011).

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8.2 Biodiversity

The term 'biodiversity' refers to the extent of genetic, taxonomic, and ecological diversity over all spatial and temporal scales (Harper and Hawksworth 1994). Most commonly, 'species richness' is used as a synonym for 'biodiversity' but 'biodiversity' in fresh waters can certainly be measured and assessed in different ways. After a short overview of size ranges within microscopically small fresh water organisms, I here discuss non-taxonomic groupings, operational taxonomic units, and species.

8.2.1 Size ranges

Viruses are biological entities and consist of a single- or double-stranded DNA, or RNA, surrounded by a protein (some viruses have a lipid coat), and are typically smaller than 100 nm, with a range of about 10–300 nm, as observed by transmission electron microscopes. However, there are viruses considerably larger than this and an example is a group of viruses found in the protozoan *Acanthamoeba*, which can be seen with a light microscope and which are 1 µm in length (Philippe et al. 2013).

If we exclude viruses, the body mass range in a typical freshwater community spans more than 16 orders of magnitude from bacteria to fish. One-third of this range is occupied by bacteria and archae, which are typically 0.2–20 µm in length. However, there are exceptions, such as *Achromatium oxaliferum*, a large sediment bacterium commonly found in freshwater and brackish environments, with cell sizes around 40 µm (Rhodes et al. 2012).

Another third of the body mass range in fresh waters is occupied by protists and microscopically small metazoans with body lengths ranging from ~ 20 µm to 2,000 µm (and six orders of magnitude differences in body weight). These organisms constitute an important part of biodiversity and energy flow in aquatic ecosystems (e.g., Reiss and Schmid-Araya 2010). Because of their small body sizes and short generation times, these organisms can respond particularly quickly to changes in environmental conditions (Finlay 2002). For instance, in planktonic communities, 'spring blooms' can result in marked changes in community size and structure as light and temperature levels rise (Gaedke 1992; Rojo and Rodríguez 1994).

Representatives of all phyla of true fungi can be found in fresh water and very common are the so-called aquatic hyphomycetes (ascomycetes and basidiomycetes). These fungi have microscopically small spores (conidia) but their hyphae can grow to substantial networks (e.g., Gulis and Suberkropp 2007) and it is difficult to assign sizes to these species.

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8.2.2 Non-taxonomic groupings

Freshwater biologists often group microscopic organisms according to their habitat (e.g., 'biofilm' (see Chapter 7), 'benthos', 'plankton', 'epibiont assemblage'). Within a habitat, these groups are further assigned to size classes and, confusingly, the range of these size bins depends on the tradition of sampling for that particular group (often determined by mesh nets used or counting techniques used).

For example, small benthic fauna are divided into the artificial groups of micro- and meiofauna, terms based on size classes for which the definitions are not unanimously agreed. Microfauna are defined as benthic organisms that are 20–200 µm in length. Meiofauna have been defined as those animals which pass a 1,000 or 500 µm sieve and are retained on a 42 µm sieve or on a 63 µm sieve (Robertson et al. 2000). Therefore, this group contains metazoans small enough to pass the upper mesh class and protozoans large enough to be retained on the denser mesh and many of them are around 200 µm in size. Some metazoan species, especially their juvenile stages, can pass the lower mesh net. Hence, many benthic species are enclosed in both the definition of meiofauna and microfauna. I suggest using the terms 'protozoans' and 'microscopically small metazoans' (micro-metazoans) when studying microscopically small animals.

Other non-taxonomic groupings include grouping organisms by their traits ('how they look'—e.g., viruses, or 'what they do'—e.g., bacteria)—such as their feeding strategy, diet, or role in the energy cycle (e.g., 'pathogen', 'symbiont', 'herbivore', 'predator', 'filter feeder', or 'shredder'). Excellent synopses exist on macroscopic freshwater invertebrates regarding their traits (e.g., Usseglio-Polatera et al. 2000); however, for microscopically small species it is necessary to consult specific taxonomic keys to find information on their traits. For example, Foissner and Berger (1996) list salinity tolerance and diet for many freshwater ciliates.

8.2.3 Taxonomy—operational taxonomic units

Genome sequencing is now a standard method to estimate biodiversity of viruses, prokaryotes, fungi, and other organismal groups and while often a species name cannot be assigned, the number of 'operational taxonomic units' (OTUs) can give an estimate of biodiversity in a sample or habitat.

Virus taxonomy is clearly an important scientific discipline for freshwater science and conservation because viruses are generally regarded as pathogens that will shape food webs (Weinbauer 2004). The majority of the viruses found in the freshwater environment are typically prokaryotic viruses (Weinbauer 2004). One way to classify viruses is by genome sequence alone (i.e., by ignoring other biological data—including host) and this approach has to date yielded thousands of species (Thompson et al. 2015) but it is not clear how many different viruses occur in particular freshwater habitats (but see Bronner et al. 2016).

Species within the bacteria and archaea are also distinguished according to their genetic similarity and the most popular metric is 16S rRNA gene sequence (deposited in GenBank and the Ribosomal Database Project). Schloss et al. (2016) point out that OTUs from aquatic environments only represent 16.5 per cent of all described, due to sampling bias towards zoonotic environments. With recent estimates of global diversity of 1 trillion species of bacteria, archae, and micro-fungi (Locey and Lennon 2016) it seems obvious that most freshwater bacteria and archaea have yet to be described. Equally, genetics (18S rDNA) are a common approach to estimate fungal biodiversity in fresh waters (Duarte et al. 2010) and OTU biodiversity estimates, depending on the method, have been shown to be higher than estimates based on microscope techniques (Duarte et al. 2010).

8.2.4 Taxonomy—species identification via microscopy

Viruses, bacteria, and protists are often classified according to their traits or morphology because consensus on how to assign species to a phylogeny or taxonomy is ongoing. It is important to know that these 'artificial' groups are in use when searching for relevant taxonomic literature and identification keys and this is especially true for protozoan species where phylogenetic classification is ever changing. Box 8.1 includes practical advice on using a microscope and the enumeration of microscopically small organisms.

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Protozoans are heterotrophic, single-celled eukaryotes and are typically assigned to either: heterotrophic flagellates (they possess flagella), ciliates (they possess cilia and at least one macro and micro-nucleus), or amoebae (they have pseudopodia). Essential taxonomic keys for these three groups are (and see references within): Foissner and Berger (1996), and Foissner (1991) for ciliates; Auer and Arndt (2001) for heterotrophic flagellates; and Page (1976 for amoebae.

When it comes to microscopic metazoans that dwell in sediments, there is rich taxonomic literature for these species (see references in Rundle et al. 2002), which is impressive as they belong to more than ten metazoan phyla in fresh water (Robertson et al. 2000). These include freshwater cnidarians and Platyhelminthes; nematodes, rotifers, gastrotrichs, and tardigrades; and tiny annelids and arthropods (such as cladocerans and copepods). Taxonomic keys to freshwater micro-metazoans that live in, and on, sediments (meiofauna) are further listed in Reiss and Schmid-Araya (2008). An online taxonomic key for a meiofaunal group is the 'Illustrated Key to Nematodes Found in Fresh Water' by the Nematology Laboratory of the University of Nebraska. The website 'plingfactory' run by Michael Plewka, is not an official key to microscopic species but gives a very impressive and accurate overview of microscopic groups in freshwater (e.g., the rotifers) and this includes prokaryotes, protists, and zooplankton.

Thorp and Covich (2009 also provide a good overview of freshwater micro-metazoans including those from the open water (i.e., small-sized zooplankton), comprising planktonic rotifers (see also Ruttner-Kolisko 1974), the planktonic cladocerans, and the open-water copepods. Many fungal species within leaf samples can be 'encouraged' to sporulate by aerating the samples. The spores can then be stained with trypan blue and their species-characteristic shape observed under the microscope and identified to species level using the key by Gulis et al. (2005).

8.3 Freshwater environments

Field- and laboratory techniques will not only depend on the types of organisms targeted but also on the freshwater system and micro-habitats sampled. In Chapter 7, lotic versus lentic environments and biofilms are discussed. Large reservoirs of freshwater biota are also found in environments such as the hyporheic zone of streams and rivers and groundwater (almost all fresh water that is not bound in ice on this planet is groundwater; see Chapter 1).

Organisms themselves are habitats and hosts for symbionts, parasites, and other hitchhikers such as epibionts. One example is the water-hoglouse *Asellus aquaticus*, whose carapax can be covered with a thick carpet of ciliates and other epibionts (Cook et al. 1998). Another obvious example are hosts of viruses as viruses all need their intracellular machinery. Lytic viruses infect cells, replicate, and then destroy cells by lysis, setting free viral progeny and cellular lysis products (Weinbauer 2004).

8.4 Sampling and assessment

A spoonful of stream sediment will sample up to 1,000 individuals of microscopically small metazoans (and many more protozoans, bacteria, and viruses) and dunking a 1 L Octoberfest

beer-glass into a pond will easily sample 100 waterfleas. Still, for a scientific sampling campaign, the total sample volume might have to be much larger if densities vary greatly in micro-habitats and if variables such as species richness are measured.

Sampling devices include Hess samplers (for sediment; see Fig. 5.3 in Anderson et al. 2013), the Bou-Rouche pump (to sample the hyporheos in the hyporheic zone), plankton nets, or the Schindler-Patalas trap and bailers (all for plankton or groundwater boreholes; see Fig. 5.3 in Anderson et al. 2013). All of these sampling techniques have to use fine mesh sizes or sample the water/sediment without any loss of water. Because microscopically small organisms are very fast colonisers of new habitats, artificial substrates, such as microscope slides (e.g., Weitere et al. 2003) or stone tiles, can be a good way to sample micro-communities.

The aims of a study and the statistical analysis that can best address the questions will determine the sampling design and sampling technique. For example, which sub-habitats are sampled, and the sample volume and sample (pseudo-) replication will depend on the questions and purpose of the study. However, all scientific studies of freshwater microorganisms will aim for replication and for the replication to yield a reliable estimate of the response variable measured (often ‘mean density’). In its simplest form, the minimum number of replicates can be estimated by performing a pilot sampling and calculating which number of replicates will yield less than 20 per cent standard error of the mean (Elliott 1977). In the case of species richness, sample number can be regressed against cumulative species richness, and when species richness plateaus the optimal replicate number has been reached.

8.4.1 Sampling viruses and bacteria

Biodiversity studies on viruses and bacteria in freshwater samples call for analysis of the genomes found, but the use of metagenetics, targeted metagenomics, and viral metagenomic

are not without controversy when it comes to the sequencing approaches. Weinbauer (2004) gives an excellent overview of methods associated with prokaryote viruses. Further examples of sampling for freshwater viruses and bacteria and their evaluation (e.g., preparation for transmission electron microscopy, epifluorescence microscopy, and molecular analyses) include Šimek et al. (2001 who sampled the meso-eutrophic Římov Reservoir in South Bohemia and Bronner et al. (2016) and Sime-Ngando et al. (2016a) who sampled viruses and prokaryotes in Lake Pavin (the latter are chapters in the book 'Lake Pavin' that includes chapters on viruses, prokaryotes, flagellates, ciliates, and fungi (Sime-Ngando et al. 2016b)). Depending on the study aim, bacteria sampling can be performed to test for the presence of certain functional groups and in this case, the samples are not treated with fixative. For example, samples can be incubated with substances utilised by the organisms and their transformation is measured. One example for this is the study by Trimmer et al. (2003) who demonstrated that anaerobic ammonium oxidation (Anammox) bacteria are present in sediments of the River Thames, UK, by incubating samples with labelled $^{15}NH_4^+$ and either $^{14}NO_2^-$ or $^{14}NO_3^-$ (or both). In this vain, Biolog-ECO microplates are a very useful tool for freshwater ecologists, where the number of functional groups present in a sample can be estimated by incubating bacteria on different substrates (in a multiwell dish) which they can either utilise or not, giving a colour reaction (Sala et al. 2006).

8.4.2 Sampling protozoans, metazoans, and fungi

Protozoans and microscopically small metazoans are as omnipresent as other microbes. Their sampling can be tricky, especially when samples have to be sieved (e.g., necessary for hyporheic samples), or when plankton nets are used, because many species can pass even very small mesh sizes and sampling with nets is only really suitable for the hard-bodied species, such as monogont rotifers and micro-crustaceans. Often a combination of sieved and

un-sieved samples is the most appropriate method (e.g., for stream benthos (Reiss and Schmid-Araya 2008)).

Freshwater hyphomycetes are abundant on decomposing leaves in running waters but spores can also be sampled from foam on the water and other micro-habitats. Sampling, culturing, sporulation, molecular approaches to sampling, and assessment of fungi in freshwater environments are described in Gulis and Suberkropp 2007; see also Wurzbacher et al. 2016.

8.4.3 Conversions and calculations: population characteristics such as production

Microscopically small organisms have vast population sizes but they are also important in terms of their biodiversity, their role in the energy cycle, their biomass, and turnover of that biomass over time (secondary production). Biodiversity estimates from different environments can be compared as taxon richness or functional richness and often it makes sense to calculate the Shannon-Wiener or the Simpson index (e.g., Reiss and Schmid-Araya 2008).

For those species, where individuals can be counted and measured, it is straightforward to estimate biomass by multiplying their average body mass by their abundance. In most cases, mass will be calculated from body dimensions and published equations (e.g., Fuhrman and Azam 1980, Reiss and Schmid-Araya 2010) and assuming a sphere shape (or the most appropriate geometric shape that resembles their body form the most). Assuming a density of 1 (or 1.1 for metazoans) will give a rough estimate of body fresh weight which can then be converted to carbon units (e.g., Reiss and Schmid-Araya 2008; Reiss and Schmid-Araya 2010). When generation times (or intrinsic rate of population increase) are known (they can

be estimated from allometric principles), then production can be calculated from biomass and temperature data (Reiss and Schmid-Araya 2010.

Fungi do not have a set body size but their biomass can be calculated by measuring the ergosterol content in a sample. Gulis and Suberkropp (2007) give a detailed overview of how fungal biomass (i.e., the amount of fungal mycelium in carbon units) can be estimated via the ergosterol extraction method (also described in Duarte et al. 2010). Pascoal and Cássio (2004) explain how to calculate spore mass.

Virus production is a very different concept compared to the secondary production of other microscopically small organisms because the amount of viruses released into the water depends on lysis, in freshwaters mostly of bacterial cells (Weinbauer 2004; also see Chapter 7 in Sigee 2004).

8.5 Bioindicators

Microorganism loading in fresh waters can indicate pollution or contamination (e.g., from soil run-off or sewage influx including pathogens) and the composition of freshwater 'micro-communities' can be used as indicators of water quality. Many microscopic species are present in fresh waters as spores (i.e., there is a 'seedbank' of non-active microbes) and due to their short generation times respond quickly to changes in their environments, such as temperature, nutrient loading, oxygen availability, or pollution. Microscopic species are present in freshwater bodies throughout the year (except for temporary meiofauna), which means they do not leave the system like insect larvae do. In addition, many species occur worldwide, meaning that indices based on these small-sized assemblages can find application across countries; which is not feasible with macrofauna.

For instance, within Europe, it is only possible to compare the presence and dominance of macrofaunal groups as opposed to individual species because these can be absent from regions within Europe (e.g., the water-hoglouse *Asellus* which dominates many British streams is absent in Portugal). Comparisons between countries are important when it comes to biomonitoring and management of fresh water on a larger scale; for example, within the European Union. For instance, the EU's 'Water Framework Directive' sought to conserve or restore freshwater bodies throughout Europe to at least 'good' ecological status by 2015 (a goal that was not reached). The EU funded the assessment of rivers and other freshwater bodies, including 'biological quality' based on macrofauna and fish (e.g., metric 'Biological Monitoring Working Party' (Hawkes 1997)). However, microbes are an omnipresent component of all freshwaters and their use in biomonitoring is explained in the following paragraphs.

A common functional measure of ecosystem heath in fresh waters is to measure the biochemical oxygen demand (BOD) as a proxy for the trophic status of the system. The BOD is essentially the 'biological response' of microscopically small organisms (often it is assumed these are mainly bacteria) to organic loading. It is the amount of dissolved oxygen used to respire organic substances in a sample. This is a semi-standardised method (defined water volume, temperature at 20ºC and darkness) used by water treatment works and governmental agencies (e.g., Environment Agency in the UK; United States Environmental Protection Agency) and often the five-day Biochemical Oxygen Demand: BOD5 is used (Delzer and McKenzie 1999).

Ciliate community composition can be used to determine the trophic status of surface freshwaters (based on the system outlined by Foissner and Berger 1996) but this approach is only used in Germany and Austria despite the fact that protozoans in general make excellent bioindicators because of their ubiquity and ease of analysis (Payne 2013). Foissner and

Berger (1996) give a brief overview of the four volumes of Foissner's ciliate taxonomy, in which they descibe typical assemblages in oligotrophic up to hypereutrophic environments. The Foissner tables include a score for a ciliate species. The scores of all ciliate species found in a system can be added up and will indicate its trophic status, similarly to other biomonitoring metrics that are based on macrofauna (e.g., Biological Monitoring Working Party (Hawkes 1997)).

Many micro-metazoans such as *Hydra* or *Daphnia* are model organisms in toxicity tests (e.g., LD_{50} to toxins). The most famous of all is the terrestrial nematode *Caenorhabditis elegans* and (unsurprisingly) evaluating the nematode community of fresh waters can indicate eutrophication. The ratio of the orders Secernentea and Adenophorea, as well as the so-called Maturity Index, are both indicators of nutrient loading in freshwaters (see Beier and Traunspurger 2001 and references therein). Similarly, freshwater hyphomycetes species are sensitive to heavy metals and nutrient loading in streams and rivers (Pascoal et al. 2010) and have equally been suggested to make excellent bio-indicators of fresh water quality (Solé et al. 2008).

8.6 Case studies

In this section, case studies are discussed to demonstrate the use of techniques and approaches associated with a particular aim: to demonstrate the abundance and activity (e.g., secondary production) of different organismal groups in the system. These case studies were chosen because they involve sampling many different microbial groups but they were mostly conduced in one or two systems only. I want to stress that, to answer general ecological questions, it is often necessary to sample many similar systems because within-system replication is essentially pseudo-replication (see Chapter 2). In other words, rather than

sampling a single stream throughout a whole year, a sampling design that involves sampling 20 streams on one sampling occasion in, for example, July only, will produce data that can answer the question. Patterns of data can then be compared between systems, generalisations can be made, and new hypotheses or models can be generated. The conclusions obtained in such a study reach beyond an observation towards estimating abundance and occupancy, testing hypotheses, formulating theory, and applying the evidence to freshwater conservation issues.

8.6.1 Ashdown Forest streams

Streams in the Ashdown Forest (South-East England), such as the Broadstone Stream or the Lone Oak, are all low-order, nutrient poor, and slightly acidic streams. The Broadstone Stream has been studied since the 1970s and is famous among freshwater ecologists. The macrofaunal community in Broadstone and Lone Oak has been described in numerous papers but two separate studies did set out to sample microbes in these systems. The overarching aim of both studies was to demonstrate that microbes are an important component of the stream community.

Jenkins et al. (2013) exposed cotton strips in 31 streams in the Ashdown Forest over seven days in summer 2011 and 49 days in winter 2012. They compared this with data from an identical study conducted in 1978 and 1979. One of their objectives was to demonstrate that the pH level in a stream determines microbial activity (i.e., to show the potential to break down organic matter in the stream). They secured 93 cotton strips to metal rods in shallow riffle sections on each occasion, harvested these, dried them, and estimated tensile strength. Those strips that easily ripped apart were decomposed the most and the value for tensile strength proofed to give a sufficient range of decomposition levels. This is not only an affordable way to demonstrate decomposition but also a very intuitive proxy for microbial

activity (in this case decomposition). Jenkins and colleagues found that decomposition increased with pH—but this pattern could only be observed in winter, not summer.

In one of these 31 streams, Lone Oak, Reiss and Schmid-Araya (2008) sampled benthic ciliates and micro-metazoans over the course of a year in monthly intervals, to show that these organisms are a diverse and abundant component of the stream community. A main focus was accurate estimates of their abundance which meant a pilot sampling was performed to estimate the number of replicates needed to get a representative mean of total abundance in a particular month (eight samples). They sampled the stream sediment with a Hess sampler, which did not have a mesh net attached but a 5 L plastic bag. The entire sample was transferred to a large bucket, the sediment was stirred, and pseudo-replicates of 50 ml were taken for ciliates. While one of these samples was simply stored on ice, the others were fixed with glutharaldeyde. The rest of the bucket sample was sieved over a 40 µm sieve and this sample was then sub-divided in the laboratory and scanned for micro-metazoans (Reiss and Schmid-Araya 2008).

The ciliates were extracted from the sediment by centrifugation. They were stained and mounted on slides using the Quantitative Protargol staining technique. Micro-metazoans were counted and identified alive. All ciliate and micro-metazoan individuals found on a sampling occasion were identified to species or the closest taxonomic level and their body length and width were measured until a total of 50 to 100 individuals was reached for each group.

Body dimensions (length and width) were converted to species-specific biovolume using published regression models (references in Reiss and Schmid-Araya 2008) and species-specific biovolumes of fixed ciliate cells were then converted into carbon content by assuming 0.14 pg C μm^3. In the case of meiofauna, body volume was converted into individual fresh weight assuming species specific gravities. The individual carbon content was estimated by assuming a dry/wet weight ratio of 0.25 and a dry weight carbon content of

40 per cent (i.e. carbon is assumed to be 10 percent of the wet weight). These conversions were necessary to show patterns of both density and biomass over the course of a year. The estimates were then used to calculate secondary production (Reiss and Schmid-Araya 2010).

The Lone Oak case study demonstrates that ciliates and meiofauna are hugely abundant (e.g., ciliates reached up almost 1 million individuals m^{-2}) and despite of 'low' standing stock of biomass, this biomass was turned over frequently; that is, secondary production was ~1 g carbon m^{-2} yr^{-1} because annual P/B ratios (production divided by biomass) for the whole assemblage exceeded 11.

These results show that ignoring microscopic metazoans and protozoans in freshwater ecology or conservation will lead to wrong conclusions about the productivity of the system and the ability of the system to attenuate and recycle nutrients, including carbon.

8.6.2 Lake Constance

While simultaneous sampling of different microbial groups is still uncommon for benthic environments (and especially running waters), there is a tradition of sampling a larger body mass range, from bacteria to fish, in the open water of lakes. A 'classic' example is the study of Lake Constance, Germany, which is known among protistologists because the ciliate community, and its role in the food web, is very well described.

Gaedke (1992) was able to demonstrate a macro-ecological pattern in Lake Constance: she found temporal variation in the biomass of small versus large plankton in Lake Constance, with shallower biomass spectra slopes in winter than in summer. She suggested this occurs because larger organisms have an intrinsic lag-time when it comes to maximising growth rates, depending on resource supply.

These observations were possible because of an extensive sampling regime that required the fixing of samples, and a focus on a large range of body sizes and groups. Plankton

abundance was measured weekly (larger phytoplankton twice a week) over the course of seven months, at the site of maximum depth and a large array of organisms, belonging to seven groups, were counted: free-living bacteria, autotrophic picoplankton, larger eukaryotic phytoplankton, heterotrophic flagellates, ciliates, rotifers, and crustaceans. The water column, from the surface to 20 m deep, was sampled with a 2-m-long tube sampler (4 L volume). Ten sequential samples were taken. Small plankton was fixed with formalin and filtered in nuclepore membranes and counted by epifluorescence microscopy with DAPI (4',6-diamidino-2-phenylindole is a fluorescent stain that binds to DNA). Larger phytoplankton including *Gymnodinium*, ciliates, and rotifers were fixed with formalin and enumerated by the Utermoehl technique (this involves letting a sample stand so the organisms can sink to the bottom of a cylinder—a microscope slide); crustaceans were counted in petri-dishes under a stereo microscope. Gaedke also describes how she estimated individual body mass from transforming body volume (which had been determined from length and width measurements and the most similar geometric shape) to carbon units, using published equations for all seven groups.

8.6.3 Římov reservoir

Šimek et al. (2001 sampled the meso-eutrophic Římov reservoir in South Bohemia (at a depth of 0.5 m) and conduced an experiment with these samples. They designed this experiment to track changes in the bacterial community due to viral lysis and flagellate grazing. This experiment involved sieving the samples into size-fractions, but one of their experimental treatments included samples that were not manipulated (not sieved). These latter samples represent an excellent case study when it comes to describing abundance and production of viruses, bacteria, and protozoans in the open water of the reservoir.

The authors used a range of techniques to target each of the three groups. Bacteria were stained with DAPI and counted and measured under an epifluorescence microscope. The size and shape of of the bacteria was used to estimate carbon content of the cells (derived from the literature), which was multiplied by abundance to get biomass estimates. Bacterial production was measured by a thymidine incorporation method and to describe the community composition, 16S rDNA sequences were analysed and *in-situ* hybridisation with fluorescent oligonucleotide probes was performed. Protozoans were fixed with Lugol and formaldehyde but instead of counting them, previous estimates on their abuncend were used. Viruses were stained and counted with both epifluoresence microscopy and TEM, the latter was also used to deteremine visibly infected bacteria and burst size. Using these methods, the authors show that viral abundaunce was as high as 5×10^7 viruses ml^{-1}; almost ten times more than the abundance of bacteria (12×10^6 bacteria ml^{-1}); and 1,000 times more than the protozoans (roughly 10^4 cells ml^{-1}). Although it was not the primary aim of this study, these estimates of abundances are important as they highlight the capacity of these organisms to flux energy (carbon) within the microbial food web (which in turn fuels higher tropic levels). For example, doubling time of this standing stock of bacteria abundance (and biomass) was estimated to be only 24 hours (reservoir water temperature was 18–20 degrees Celsius) which explains the high abundances of protozoans feeding on them.

Aquatic ecologists need these type of estimates to compare systems. The microbial community in the Římov reservoir displays a classic pyramid of numbers where the small are more abundant than the large organisms. Theoretical ecology is using the relationship between the range of organismal sizes and abundance (imagine a classic abundance pyramid flipped to the right by 90 degrees) to compare ‘healthy’ systems with those that are impacted by human activity (see Petchey and Belgrano 2010).

8.7 Conclusion

Both the scientific community and conservation bodies are now aware of the fact that, in order to judge ecosystem health, we need the tools to measure abiotic factors as well as the biota present in these systems. Molecular techniques, as well as a long tradition of publications by dedicated taxonomists and freshwater ecologists, make it possible to accurately assess microscopically small organisms and to compare these communities across systems—and to therefore judge ecosystem health. Microbes have the largest size range within fresh waters, the highest abundances, the highest species richness, and are the main drivers of important ecosystem processes such as whole-system respiration: they truly rule fresh waters.

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Box 8.1 Microscope and enumeration techniques

Biologists studying microscopically small organisms rely on microscopes when it comes to studying external or internal features of microbes (e.g., for taxonomy or enumeration; see Chapter 7). While viruses and many bacteria are so small that transmission electron microscopes (TEM; e.g., Šimek et al. 2001) are used for their identification and enumeration (see Chapter 7); it is possible to identify and count many prokaryotes and microscopic eukaryotes with light microscopy.

Light microscope types used include: the inverted microscope, the compound microscope (this includes the epifluorescence microscope) and the stereo-microscope. The latter is useful

for enumeration of the ‘larger’ eukaryotes such as larger meiofauna or zooplankton but for taxonomy the maximum magnification (up to × 400) is generally too low for species identification. Species that are over 40 micrometres in length can usually be identified with × 400 and × 600 magnification using a compound- or inverted microscope (using the × 10 ocular and the × 40 or × 60 objective, see Chapter 7). Differential Interference Contrast (DIC, also called Normarski) and Phase Contrast can be very useful microscope features—especially for the unicellular eukaryotes.

Microscopic pro- and eukaryotes are so small and abundant that it will be necessary to bring samples back to the laboratory to identify taxa and count their numbers. USB microscopes, however, are useful tools in the field when it comes to counting macroscopic fauna.

Fixing-, sedimentation-, filter mounting-, and staining techniques are described or referenced in the examples in Table 8.1; however it is worth noting that many microscopic species either cannot be fixed or not identified when they are dead. For example, bdelloid rotifers are best observed alive so it is possible to count the number of toes and then fix them to view their jaws (called trophi).

To study living protozoans and metazoans, individuals have to be transferred individually from a sample into a small drop of water on a microscope slide using a pipette or an eyelash glued to a pipette (the latter is used for nematodes) so they can be ‘squeezed’ with a microscope slip that has Vaseline ‘feet’ (see Foissner and Berger 1996) which both slows the individual down and gives a much better microscope image, allowing to use the higher objectives (up to × 1,000 magnification); for example, ciliates (Foissner and Berger 1996).

If the aim of the study does not call for detailed taxonomy, enumeration can be automated; for example, by cell counters. Enumeration techniques of bacteria include flow

cytometry (see Chapter 7). Automated image analysis is very common for plankton samples but not so much for benthic samples where the organisms have to be separated from biofilm or sediment particles (with a pipette, a needle, or by centrifugation techniques with a medium denser than water—such as Lugol).

When using a light microscope, simple microscope slides can be used for enumeration purposes but often specially designed counting chambers are used to estimate abundances in a water sample. The Sedgewick Rafter Counting Chamber (gridded and holds 1 ml of water; see Chapter 7) is an excellent example for the latter and useful for counting organisms as small as flagellates up to the larger micro-metazoans. For larger meiofauna and zooplankton, petri dishes that are used with a stereomicroscope can be more useful because they hold a larger volume of water.

Table 8.1 Taxonomic guides or references to the main groups of microorganisms reviewed in this chapter

Group	Sub-group	Reference
Viruses	Operational taxonomic units	Weinbauer 2004
Prokaryotes	Operational taxonomic units	Duarte et al. 2010
Fungi	Operational taxonomic units	Duarte et al. 2010
	Fungal species	Gulis et al. 2005
Protozoans	Ciliates	Foissner and Berger 1996; Foissner 1991
	Heterotrophic flagellates	Auer and Arndt 2001
	Amoebae	Page 1977
Microscopic metazoans	Meiofauna (sediment metazoans)	Rundle et al. 2002; Reiss and Schmid-Araya 2008
	Zooplankton (open water metazoans)	Thorp and Covich 2009; Ruttner-Kolisko 1974

Table 8.2 Literature that explains either sampling method, sample processing, or counting of microscopically small organisms. Examples are given for particular freshwater systems.

Group	**System**	**Reference**
Viruses	Lake	Bronner et al. 2016
Prokaryotes	River	Trimmer et al. 2003
	Groundwater	Griebler and Lueders 2009
Fungi	Stream	Gulis and Suberkropp 2007
	Lake	Wurzbacher et al. 2016
Protozoans	Sediment	Reiss and Schmid-Araya 2008
	Open water	Scherwass et al. 2010; Kammerlander et al. 2016
	Artificial substrates	Weitere et al. 2003
Microscopic metazoans	Sediment	Reiss and Schmid-Araya 2008
	Open water	Kammerlander et al. 2016